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PRINCIPAL INVESTIGATOR: Beatrice S. Knudsen, M.D., Ph.D.

CONTRACTING ORGANIZATION: Cornell University Medical College
New York, New York 10021

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13. ABSTRACT (Maximum 200 Words) The scope of this grant proposal is to investigate whether adhesion of prostate cancer cells to extracellular matrices regulates cell growth and survival. We have shown that attachment of LNCaP cells to a variety of matrices results in the phosphorylation of the focal adhesion kinase, FAK. However the downstream signaling pathway to Crk is not activated. This is due to the lack of Src activation upon cell adhesion. Despite the absence of Src activation, adhesion to the extracellular matrix stimulates cell proliferation, but not cell migration. In summary, we have demonstrated that Src activation is critical for cell migration and for the assembly of the p130Cas/Crk pathway in prostate cancer cells. This information will help in the design of drugs that can inhibit prostate cancer metastasis.			
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INTRODUCTION:

Adhesion of prostate cancer cells to extracellular matrix proteins regulates cell proliferation and cell survival (1). We therefore proposed to investigate the basic mechanisms that are responsible for tumor growth as a consequence of prostate cancer cell adhesion to different matrices.

Integrins function as adhesion receptors at the cell – matrix interface through their binding to extracellular matrix proteins (2). Therefore signaling pathways that promote cell proliferation and survival originate from the cytoplasmic domain of integrins and associated molecules (3). Binding of cells to matrix proteins elicits the transmission of signals from the cell surface to the nucleus and regulates the cell cycle and the transcription of genes related to cell survival (4). The focal adhesion kinase (FAK) is intimately associated with activated integrins and through binding and phosphorylation of its substrates FAK initiate several signal transduction pathways (5). One of these pathways involves the scaffolding protein, p130Cas (6-8). The central role of p130Cas in oncogenesis has been well documented in *Src* and *Crk* transformed cells (9). Therefore the phosphorylation of p130Cas in response to cell adhesion and the activation of downstream signaling pathways constitutes the main goal of this grant proposal.

BODY:

Research accomplishment for task 1: To determine the involvement of Crk (CT10 related kinase) proteins and their ligands in b1-integrin mediated signal transduction events.

- **Determine which proteins become tyrosine phosphorylated in LNCaP (prostate cancer cells) cells upon cell adhesion and bind the CrkSH2 domain in a time course experiment:**

We have analyzed the adhesion of LNCaP cells to the extracellular matrix of primary human bone stromal cells. In these conditions, the focal adhesion kinase, FAK is activated and its phosphotyrosine content increases. However, our experiments demonstrate, that these cells have a defect in the activation of the cytoplasmic tyrosine kinase, *Src*. Therefore downstream pathways that lead to Crk/ CrkL (Crk-like) binding are insufficiently activated. This results in a profound defect in cell migration.

- **Analyze Crk containing complex with regards to their individual protein constituents:**

We identified the focal adhesion protein, paxillin (10), as the only protein that could bind the CrkSH2 (Src homology 2 domain) (11, 12) domain in LNCaP cells after adhesion to bone matrix. Paxillin localized to the focal contacts between LNCaP cells and the bone matrix (appendix, figure 1).

- **Determine differences between c-CrkII and c-CrkL complexes:**

We have confirmed CrkII, CrkI and CrkL expression in LNCaP cells. Those are the three ubiquitously expressed Crk-family members (13). Precipitation of Crk or CrkL in LNCaP or TSU cells shows that p130Cas co-precipitation is greatly increased in the TSU cells compared to LNCaP cells (appendix, figures 2 and 3). In contrast, paxillin binds to Crk in both cell lines (appendix, figure 3). There is no apparent difference between Crk and CrkL binding proteins in TSU or LNCaP cells.

- **Set up a reproducible adhesion mediated proliferation assay for LNCaP cells:**

We have demonstrated that the extracellular matrix from bone cells promotes adhesion (appendix, figure 4) and stimulates proliferation (appendix, figure 5) of LNCaP cells. Comparison with other extracellular matrices demonstrated a greater mitogenic potential in the matrix from human bone cells compared to mouse bone cells or human osteosarcoma cell lines. It is not clear which matrix constituent stimulates cell proliferation (appendix, figure 5).

- **Establish an anoikis assay for suspended LNCaP cells:**

We have shown that LNCaP cells undergo apoptosis when kept in suspension. DNA laddering was observed 24 hours after the lack of adhesion and increased over 72 hours.

- **Perform measurement of anoikis induced activation of JUN kinase:**

We were unable to link the Crk pathway to JUN kinase and anoikis (apoptosis caused by a lack of cell attachment) in LNCaP cells, since these cells have a fundamental defect in Src activation. Recent data have demonstrated, that these cells use the Akt pathway as major activator for cell survival (14). Assembly of the Crk pathway may therefore not be the only pathway required for cell survival in the LNCaP cell line.

- **Measure the augmentation of Rap1A (Ras-like GTPase) activation upon cell adhesion or detachment:**

We have obtained the glutathione-S-transferase fusion protein to specifically precipitate Rap1A from LNCaP cell lysates (15). The baseline level of activated Rap1A is high and we could not evaluate a change in Rap1A activation using our assay system. Development of a more quantitative assay is warranted, however this exceeds the scope of this grant proposal.

Research accomplishment for task 2: To determine the functional role of the Crk proteins during adhesion mediated survival of prostate cancer cells.

- **Establish the MAPK (Mitogen activated protein kinase) assay to measure integrin induced activation of the Ras pathway:**

Using the recently available phospho-MAPK antibody, we demonstrated that adhesion to matrix alone does not lead to activation of MAPK. This is a sensitive Western-blot assay, but it is uncertain whether the detection limit of the assay correlates with the true intracellular functional MAPK. It is possible that a small amount of activated MAPK may not be detectable by the assay. It is also possible that the LNCaP cells secrete small amounts of autocrine growth factors and that cell adhesion increases the activity of these growth factors. These growth factors will then induce cell proliferation.

- **Generate stable transfected LNCaP cell lines that express C3G (Exchange factor for Rap1A) and CrkL:**

We detected high expression of CrkL and C3G in the LNCaP cells (appendix, figure 2). Therefore these proteins are not limiting in the Crk/CrkL pathways. Since inaddition, this pathway is not activated we decided not to generate of C3G or CrkL overexpressing lines.

- **Measure complex formation of Crk in transfected cell lines:**

See above statement why such cell lines were not generated.

- **Measure JUN kinase activity upon adhesion in Crk overexpressing LNCaP cells and relate to apoptosis or anoikis:**

We have demonstrated JUN kinase activation in anisomycin treated LNCaP cells as a positive reference standard using an in-vitro kinase assay (16). However, we were unable to demonstrate JUN kinase activation by this assay upon cell detachment. It is possible that activation of JUN kinase is low, or that other apoptotic pathways are activated.

- **Measure Rap1A activities in Crk overexpressing cell lines and correlate with growth arrest:**

Recent evidence points to a role of Rap1A in stimulating cell proliferation and not in inducing growth arrest of epithelial cells (17). This is different from earlier studies where Rap1A was found to be antagonistic to Ras in fibroblasts. In LNCaP cells it has now been demonstrated that activation of Rap1A can lead to MAPK activation. Therefore, Crk activation could stimulate cell proliferation and not induce growth arrest in LNCaP cells. Consistent with this hypothesis, we found that inhibition of the CrkSH3 domain in TSU prostate cancer cells is “toxic”, thus inhibit cell proliferation or cause apoptosis.

- **Express a mutant Crk protein in LNCaP cells**
- **Construct a FAK-C3G fusion construct for expression in LNCaP cells**
- **Confirm localization and functional activity of the expressed fusion protein**

These aims were not pursued, since the FAK/Crk/C3G pathway appeared not to be activated in LNCaP cells.

Research accomplishment for task 3: Determine the utility of FAK and p130Cas expression and phosphorylation in human prostate cancer tissues.

- **Establish a process whereby phosphorylation of cytoplasmic proteins is preserved**
- **Microdissect tissue**
- **Establish ELISA assay with prostate cancer cell lines to obtain a standard curve:**

These aims will be pursued, once the role of FAK and p130Cas in prostate cancer is better understood.

- **Submit additional prostate cancer specimens to the tumor bank:**
We have collected a total of 35 cases of prostate cancer. A frozen section was performed to ensure the presence of tumor in the frozen tissue. This has increased the chance of tumor to >90% from originally 30%. We have also established a culture system for primary prostate epithelial cells in which we are analyzing the Crk pathway.

- **ELISA for prostate tumor tissue:**
This aim is awaiting the completion of characterization of the Cas/Crk pathway in other prostate cancer cell lines available.

- **Clinical correlation and statistics:**
A clinical database accompanies the tissue that was deposited into the tissue bank. This will constitute a valuable resource for future pilot studies regarding the clinical role of signal transduction proteins in prostate cancer.

RESEARCH ACCOMPLISHMENTS:

We set out to demonstrate the role of the Crk/Cas pathway in the adhesion mediated survival of LNCaP cells. However, we realized that this pathway is not activated upon adhesion of LNCaP cells to extracellular matrix. Consequently, the main accomplishment of this project is the discovery of the defect in LNCaP cells that leads to reduced cell adhesion and migration. This defect lies in the proper activation of the Src kinase. The reason for this remains unknown, but it is most likely that LNCaP cells fail to express critical adhesion receptors. The results demonstrate the role of Src in cell migration, which is an important mechanism in the generation of tumor metastasis. Thus Src inhibitors are now tested as antimetastatic drugs, in-vitro and in animal models of tumor metastasis.

- Generation of a biologically relevant system to analyze the interaction of prostate cancer cells with bone.
- Identification of critical mechanisms that are required for cell migration of prostate cancer cells.
- Demonstration of FAK and p130Cas phosphorylation and complex formation with downstream effector proteins by co-immunoprecipitation in TSU prostate cancer cells
- Proliferation assay for matrix adherent LNCaP cells through incorporation of BrdU.
- Demonstration of mitogenic properties of individual extracellular matrix proteins and intact matrices on LNCaP cells
- Anoikis assay and Jun-kinase activation assay
- Assays for activation of Rap1A in LNCaP cells
- Procurement of prostate cancer tissue for future research

REPORTABLE OUTCOMES:

- A manuscript entitled "Adhesion of LNCaP cells in the extracellular matrix of bone stromal cells" has been submitted.
- A manuscript entitled 'Normal and Malignant Prostate Epithelial Cells differ in their response to HGF/SF stimulation' has been submitted.
- The proposed work will provide the basis for submission of an RO1 grant in March 2001.

CONCLUSIONS:

Adhesion of LNCaP cells to a variety of matrices results in the phosphorylation of the focal adhesion kinase (FAK). However, the downstream pathway that involves the phosphorylation of the scaffolding protein, p130Cas, is not activated. This is because of a lack of Src activation in the LNCaP cells. Because p130Cas is not phosphorylated, it does not bind Crk/CrkL. It is most likely that the LNCaP cells utilize signalling pathways other than Cas/Crk for adhesion dependent cell survival. These would include the PI3-kinase/Akt pathway. Attachment of LNCaP cells to extracellular matrix proteins also stimulates cell proliferation. The original hypothesis, that cell adhesion of LNCaP is critical for survival and proliferation is still valid. However, the Cas/Crk pathway is important for cell migration, but not for cell survival in the LNCaP cells.

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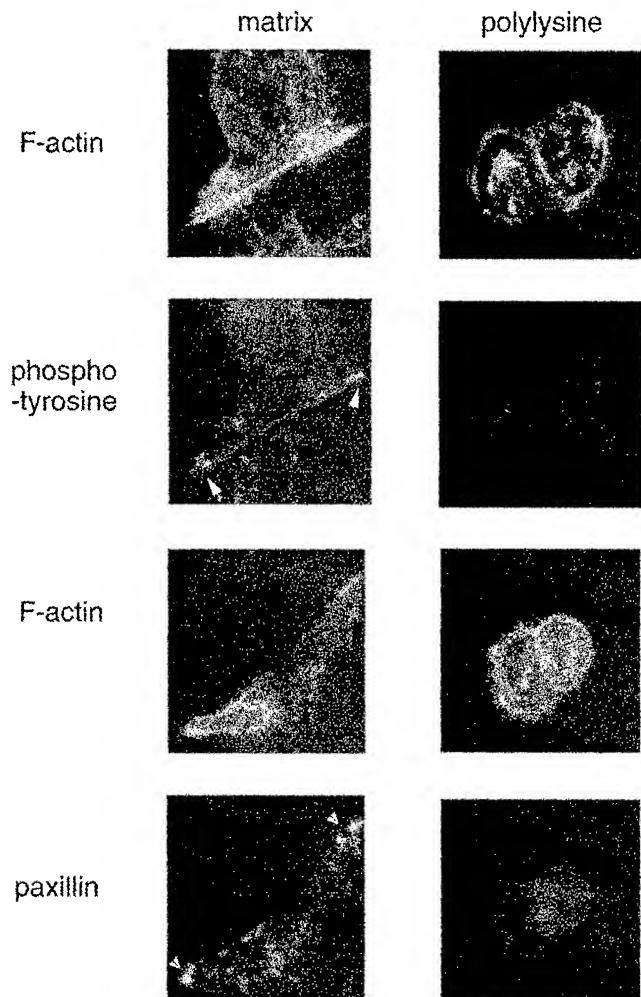


Figure 1: Localization of tyrosine phosphorylated proteins and paxillin to focal contacts.
LNCaP were either adhered to hBdC-matrix or polylysine. Adherent cells were first stained with the 4G10 anti-phosphotyrosine antibody or anti-paxillin and a fluoresceine conjugated anti-mouse secondary antibody, followed by staining with rhodamine-phalloidin. Confocal images obtained with the "green" and "red" Helium-Neon lasers were overlayed. The arrows point to paxillin, whereas the arrow heads indicate tyrosine phosphorylated proteins at cell-matrix contact sites.

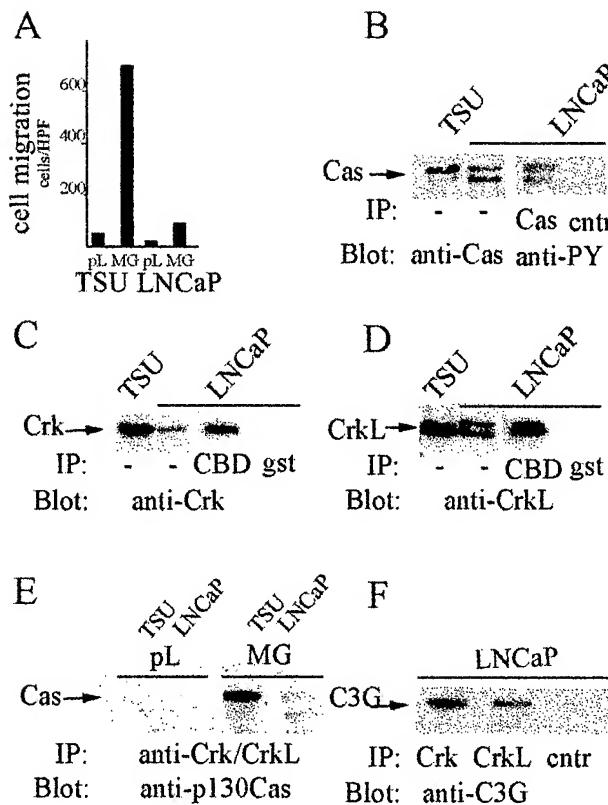


Figure 2: Cell migration and Crk/CrkL complexes in LNCaP and TSU-Pr-1 cells. **A.** Migration of TSU-Pr1 and LNCaP cells through matrigel coated Transwell™ filters. Migration was quantified by counting the cells that migrated through the filter. The bars depict the mean number of cells per field at 100X magnification that migrated through the filter. **B.** Expression of p130Cas and CasL in TSU-Pr1 and LNCaP cells. The first two lanes represent a straight Western blot with 2 ug whole cell lysate, the third lane shows tyrosine phosphorylation of both bands in LNCaP cells. **C and D.** Expression of Crk and CrkL in TSU-Pr1 and LNCaP cells. The first two lanes show a straight Western blot with 20 ug of whole cell lysate, whereas the third lane shows Crk protein precipitated with a Gst-fusion protein of the C3G-Crk binding region that specifically binds to the CrkSH3 domain (Gst-CBD). The last lane contains the Gst control. **E.** In-vivo Cas-Crk/CrkL complex formation. 300ug of lysate from TSU-Pr1 and LNCaP cells adhered either to polylysine (pL) or matrigel (MG) was precipitated twice with Crk/CrkL. The entire precipitated protein was analyzed for bound p130Cas protein by Western blotting. **F.** Crk and CrkL bind C3G in the cytoplasm. 0.5 mg of cell lysate was precipitated with anti-Crk or anti-CrkL antiserum. Blots were probed with anti- C3G.

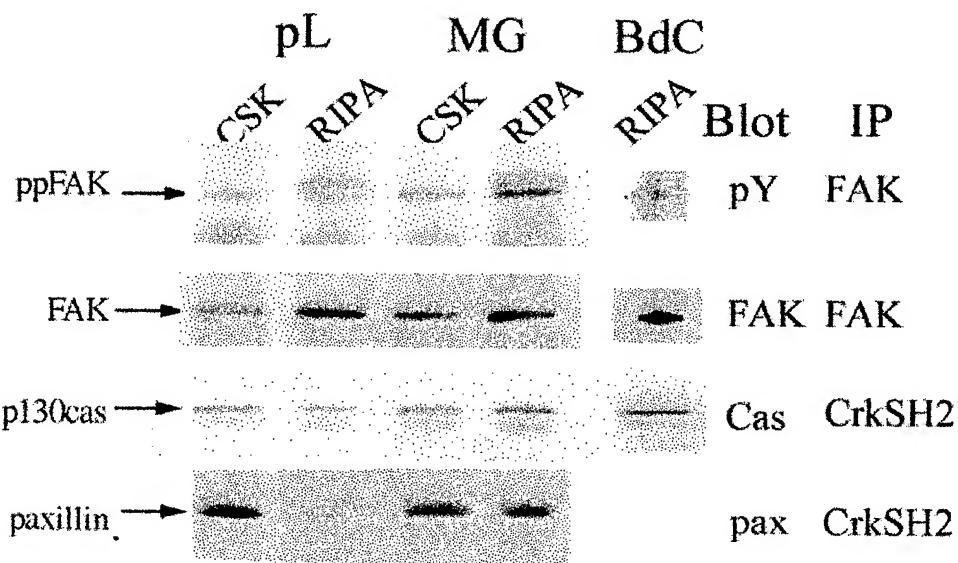


Figure 3: Phosphorylation of FAK and FAK binding proteins in LNCaP cells. LNCaP cells were adhered to polylysine (pL), matrigel (MG) or hBdC-matrix (BdC). After 7-8 hours adherent cells were first extracted with CSK buffer followed by extraction with RIPA buffer. 500ug of cell lysate was precipitated with anti-FAK or with a Gst-CrkSH2 fusion protein. Western blots were probed as indicated and bands visualized by chemiluminescence. The same blot was probed with anti-phosphotyrosine and anti-FAK. The identity of paxillin was confirmed by comigration with immunoprecipitated paxillin from the same cell lysates.

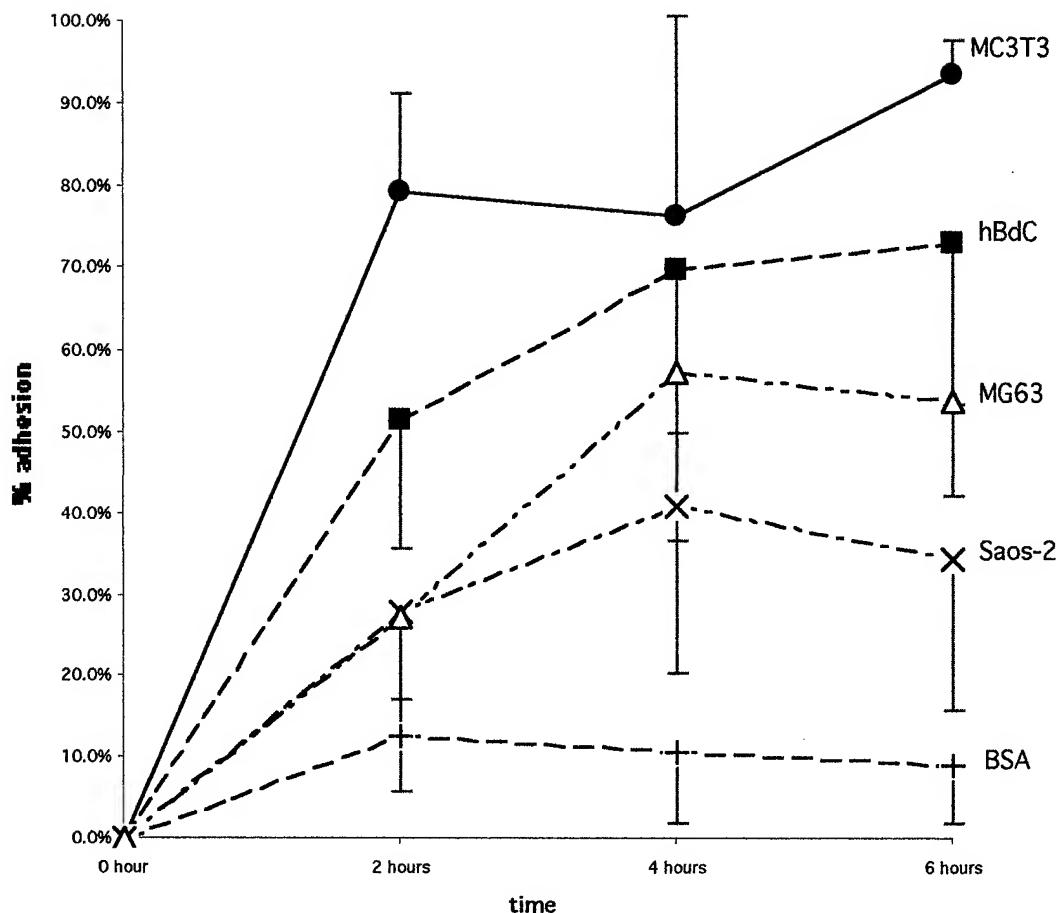


Figure 4: Adhesion of LNCaP cells to matrices from bone derived cells
Extracellular matrices synthesized on 6cm tissue culture plates by mouse
osteoblast MC3T3 cells (MC3T3), human bone-derived cells (hBdC)
and the human osteosarcoma cell lines, Saos-2 and MG63 were prepared
and incubated with 3×10^6 LNCaP cells per dish for the indicated amounts
of time. At each time point the total protein of suspended and adherent
cells was determined from duplicate plates. The % adhesion was
calculated by dividing the mg adherent protein by the total (adherent and
suspended) mg protein. Each data point represents the mean of 5
independent experiments, with error bars showing the standard deviation
of mean.

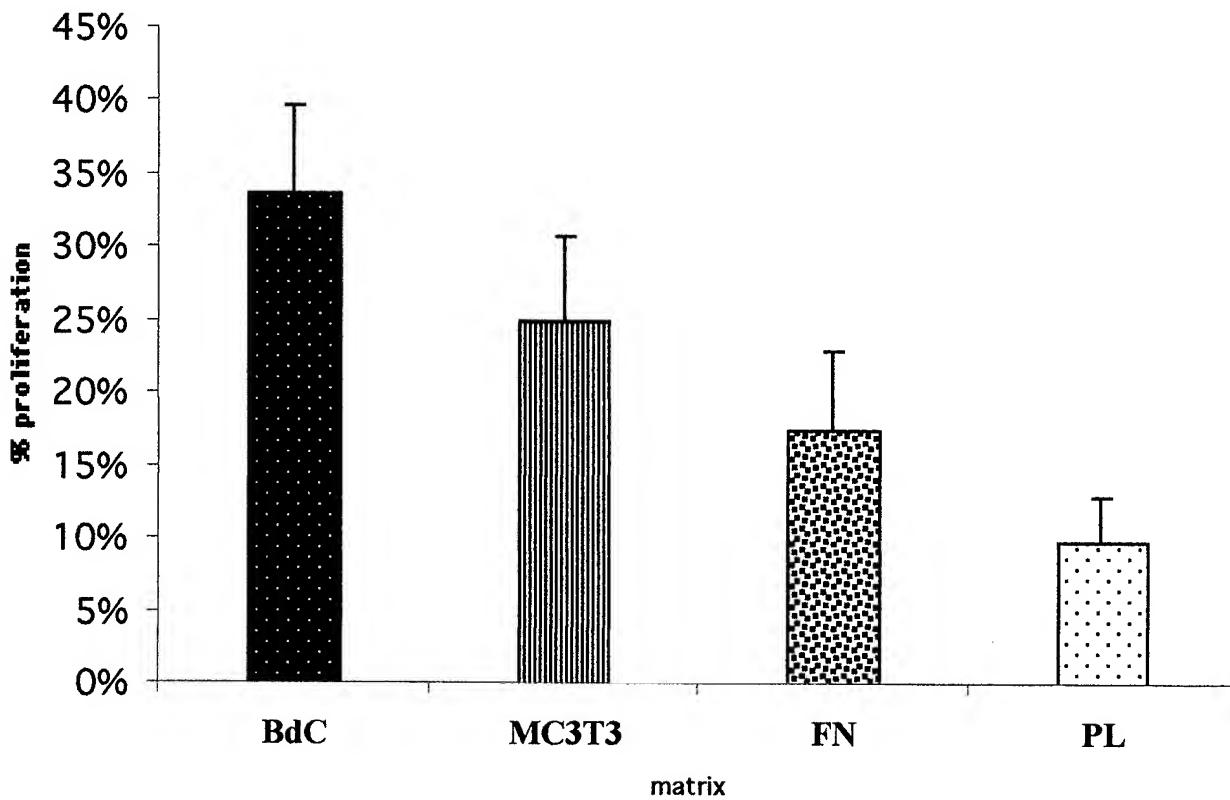


Figure 5: Proliferation of LNCaP cells on matrices and matrix constituents. LNCaP cells were serum starved for 72 hours, trypsinized and plated on the coverslips coated with matrix from human bone-derived cells (hBdC), MC3T3 mouse osteoblasts (MC3T3), fibronectin (FN) or polylysine. 12 hours post plating, BrdU was added to the wells for an additional 12 hours. Duplicate coverslips were then fixed in methanol:acetone (1:1) and stained for BrdU incorporation with the anti-BrdU monoclonal antibody as described in the Material and Methods section. Approximately 300 cells were counted per coverslip. The bars represent the mean of at least three separate experiments with the error bar indicating the standard deviation of the mean.